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13. ABSTRACT *(Maximum 200 words)*

The general objective of this project was to examine some of the lipid dependent processes that influence breast cancer development, so that more effective strategies can be applied to the prevention and control of this disease. Previous studies have indicated that low fat diets, and those containing large amounts of omega-3 polyunsaturated fatty acids (PUFA) [e.g. menhaden oil], do not enhance breast cancer development in rodents as effectively as those containing large amounts of omega-6 PUFA [e.g. corn oil]. This project sought both to evaluate whether diets containing various blends of omega-3/ omega-6 PUFA at several intermediate ratios could effectively delay tumor development, and to determine whether these dietary tumor effects were at all correlated with alterations in insulin like growth factor (IGF) metabolism. Our results, have indicated that several of the blended diets containing moderately high omega-3 content were effective in delaying tumor development, but there appeared to be no direct statistically significant correlation between those differences and the circulating levels of IGF-1 as measured by radioimmunoassay, or tumor messenger RNA levels for IGF-1, IGF-2, IGF-1 receptor, or IGF-2 receptor as measured by ribonuclease protection assay.

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William J. Coats — 1/27/98

PI - Signature — Date

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INTRODUCTION:

This report summarizes the results obtained from the complete period of grant funding. During this time three individual dietary experiments were carried out; an initial study involving R3230AC transplanted tumors (1994-5), and two others evaluating the effects of dietary lipids on NMU induced mammary tumors (1995, 1996). In 1997, total RNA was extracted from selected tumors in each dietary treatment groups, and subjected to RNA protection assays to quantify the amount messenger-RNA for IGF-1, IGF-2, IGF-1 receptor, and IGF-2 receptor present.

BODY:

Our initial attempt to induce mammary tumorigenesis in female F-344 rats with NMU was unsuccessful. The F-344 rats in the different diet groups did not develop sufficient tumors for group analysis. The reasons for this lack of success is unclear in light of the published work of others. However, in later experiments using Sprague Dawley rats, we were able to obtain successful results.

The component of the experiment studying F-344 rats implanted with R3230AC tumor transplants was successfully completed. In this experiment, 28 week old female rats received two tumor implants in their mammary fat pads. They were then divided into 4 diet groups: 20%CO, 5%CO, 4%FO+1%CO, and 19%FO+1%CO. The tumors grew rapidly in all rats and they were killed 21 days following tumor transplantation. Their tumors were removed, weighed, and their membrane fatty acid profiles determined by gas chromatography. Representative fatty acid profiles are shown in Figure 1, and demonstrate that the different diets did induce important alterations in their tumor membrane fatty acid compositions. Table 1 presents the weights of the tumors from the different diet groups.

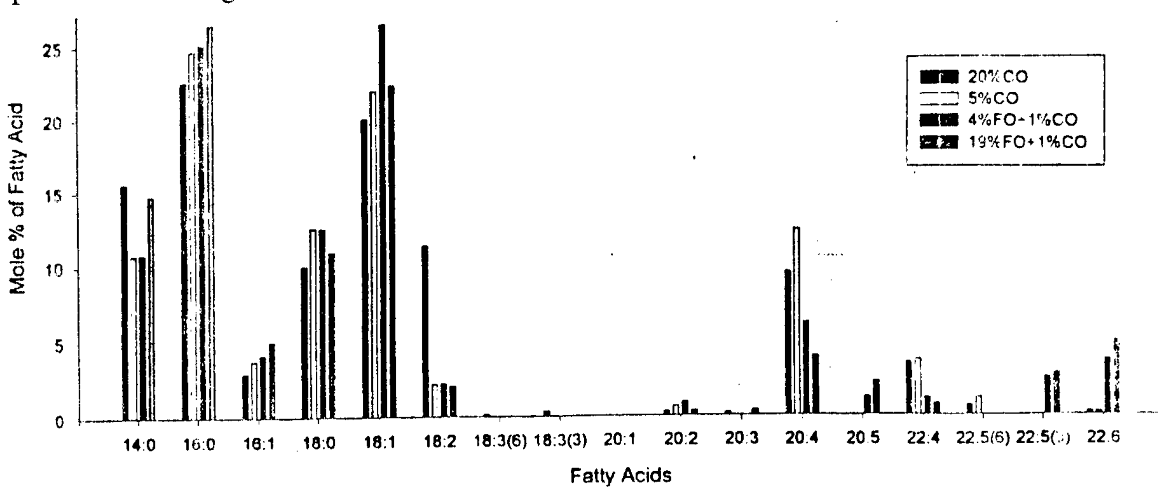


Figure 1. **The fatty acid composition of R3230AC tumor microsomes obtained from each diet group. The moles of each fatty acid present are represented as a percentage of the total lipid content.**

Table 1. These data indicate the tumor mass removed from the number of animals(n) in each diet group.

R3230AC Tumor Burden
(Mean$\pm$ S.E.)

Diet Group	**20% CO**	**5% CO**	**4%FO+1%CO**	**19%FO+1%CO**
n	4	5	4	6
Tumor Wt.	4.617	4.806	4.220	3.00
	$\pm$ 0.396	1.358	1.137	0.790

These results indicated that there was a trend toward a reduced tumor burden in the rats fed a high fish oil diet relative to the high corn oil diet, but this difference did not reach statistical significance.

In 1995 we began our first study examining the development of n-methyl nitrosourea (NMU) induced mammary tumors in female Harlan Sprague Dawley rats. These rats received an IV infusion of aqueous NMU (5mg/100g body weight) at 50 days of age. Afterwards, they were placed on their individual semisynthetic diets, and then monitored for tumor development. The diets were prepared in the vivarium diet kitchen using analyzed ingredients of uniform quality. The corn oil (omega-6 PUFA)was obtained commercially(ICN/Teklad) and the menhaden oil (omega-3 PUFA) was obtained from the NIH/NOAA biochemical test material program(Southeast Fisheries Science Center, Charleston SC). The oils were adjusted with the antioxidants alpha tocopherol, gamma tocopherol, and tertiary butylhydroquinone in order to maintain equivalent levels in both oils. The diets were equicaloric in the respective high and low fat diet groups, and appropriate adjustments were made for protein, mineral, and vitamin content. The time of appearance of the first palpable tumor was recorded, and when a tumor's size exceeded 2 cm in diameter the host was killed, the sera collected, and the carcass autopsied. The tumor latent period was defined as the duration of time from NMU administration until death, and the tumor burden was defined as the total weight of the tumor tissue obtained at autopsy. All sera and tumor tissue were stored at -70 degrees centigrade until used for specific assay. The lipids extracted from tumor microsomal membranes were profiled by gas chromatography. Serum IGF-1 determinations were made using a radioimmunoassay kit (Nichols Institute Diagnostics). All serum samples from the experiment were evaluated simultaneously in a single batch analysis.

Figures 2 and 3 present the representative fatty acid profiles the tumor membranes from these animals in each diet group. Table 2 and Figures 4-8 present the data on tumorigenesis and the serum IGF level obtained for each diet group. All values are presented as mean values. The number [n] or individual analyses is that of the group unless specifically noted.

Figure 2. These are the gas chromatographic profiles of the fatty acids extracted from the tumor mircosomes of each diet group. The moles of each fatty acid present are represented as a percentage of the total lipid content of the membrane(Mean + S.E.).

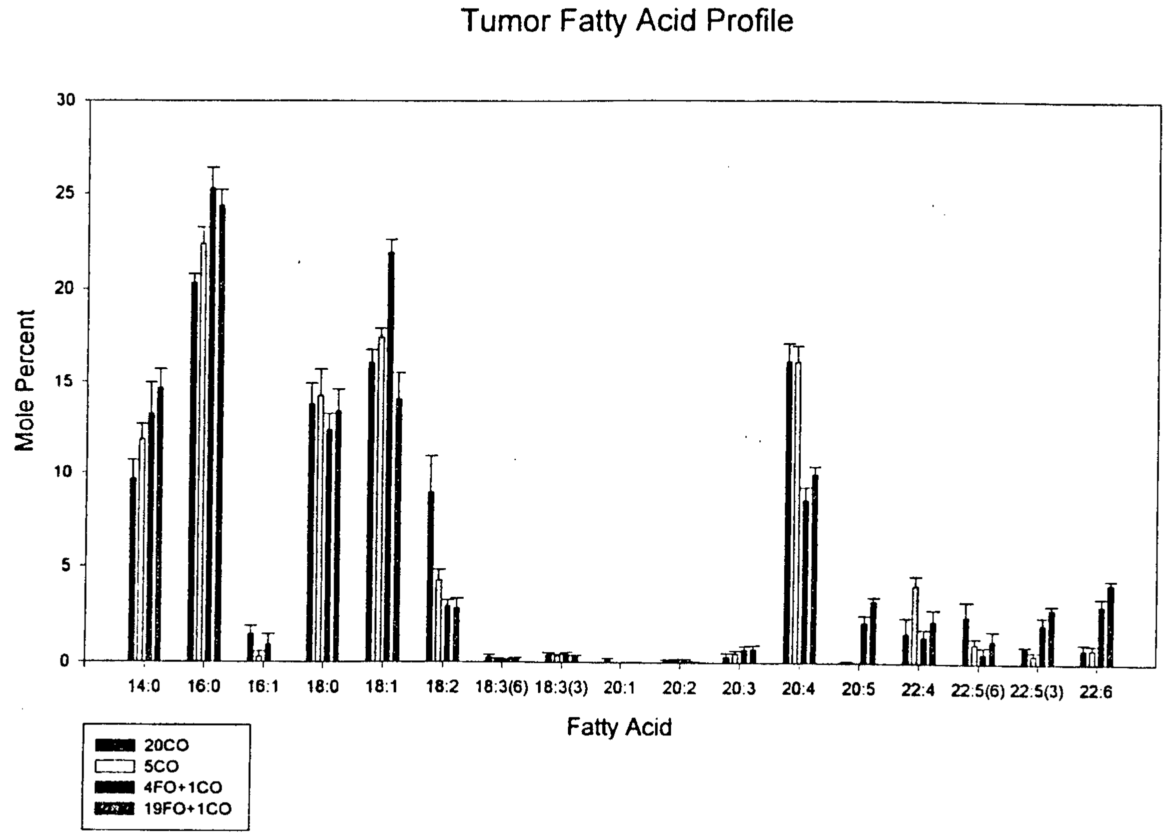


Figure 3. This figure specifically highlights the omega-6 (18:2, 20:4) and omega-3 (20:5, 20:6) differences (Mean $\pm$ S.E.) in the fatty acid profiles among the different tumor microsomes.

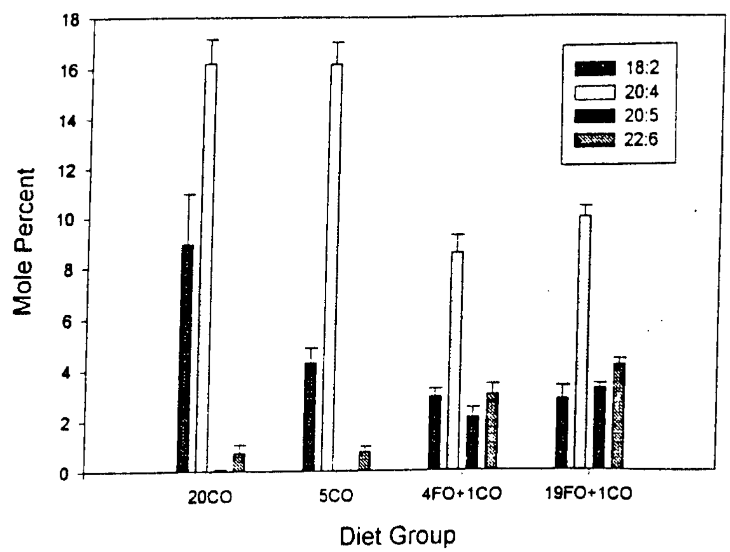


Table 2. This table provides the autopsy data and serum IGF-1 levels of the animals in each diet treatment group

NMU Induced Tumorigenesis
(Mean ± S.E.)

Diet Group	[n]	First Tumor Appearance (days)	Tumor Latency (days)	Tumor Burden (grams)	Tumor Number	Serum IGF-1 ng/ml
20%CO	11	78.64	117.36	7.07	3.18	726.40
		± 8.72	9.62	4.01	0.52	96.98
5%CO	11	85.18	116.36	3.77	1.82	731.80
		± 8.27	8.97	2.88	0.30	68.51
4%FO+1%CO	12	101.00	122.83	5.17	2.92	678.60
		± 7.91	6.34	2.94	0.40	82.23
19%FO+1%CO	12	78.90	111.66	6.53	3.33	802.40
		± 4.50	6.69	2.51	0.38	62.86

Figure 4. This figure shows the time from NMU administration to first tumor appearance in each diet group (Mean ±S.E.).

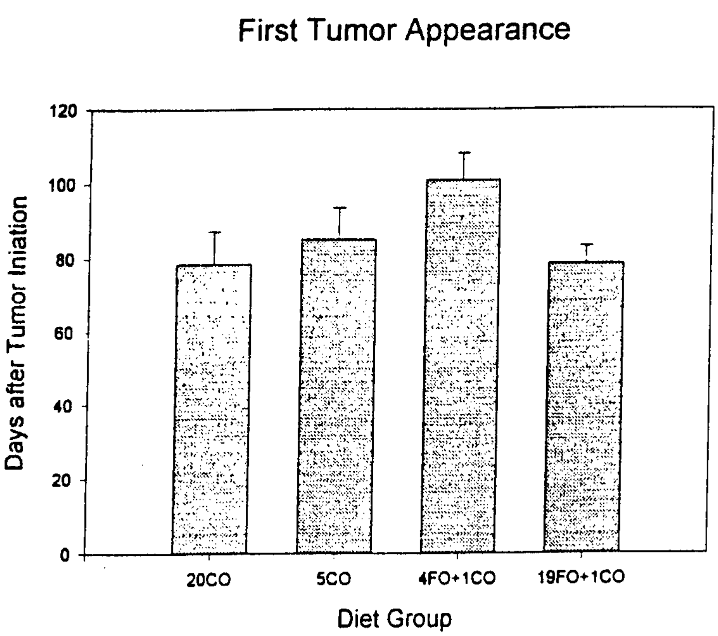


Figure 5. This figure shows the time from NMU administration to time of sacrifice for each diet group (Mean ±S.E.).

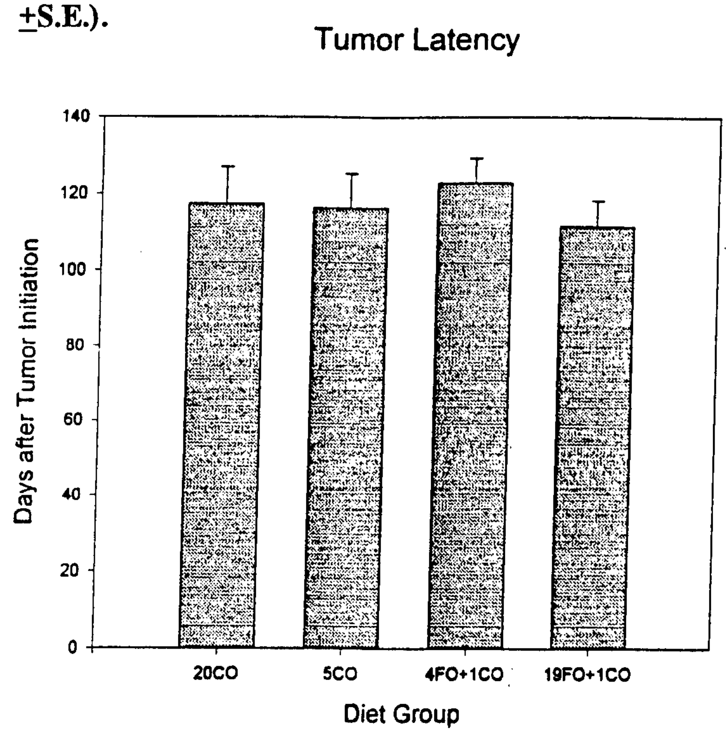
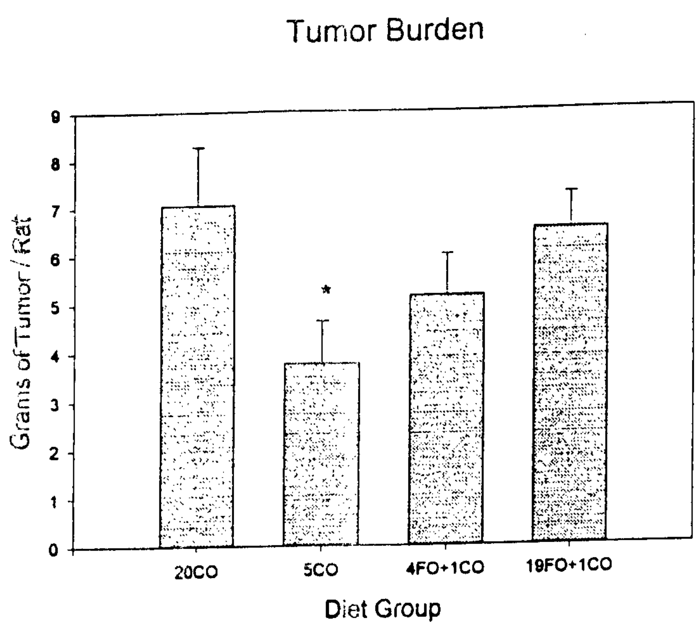
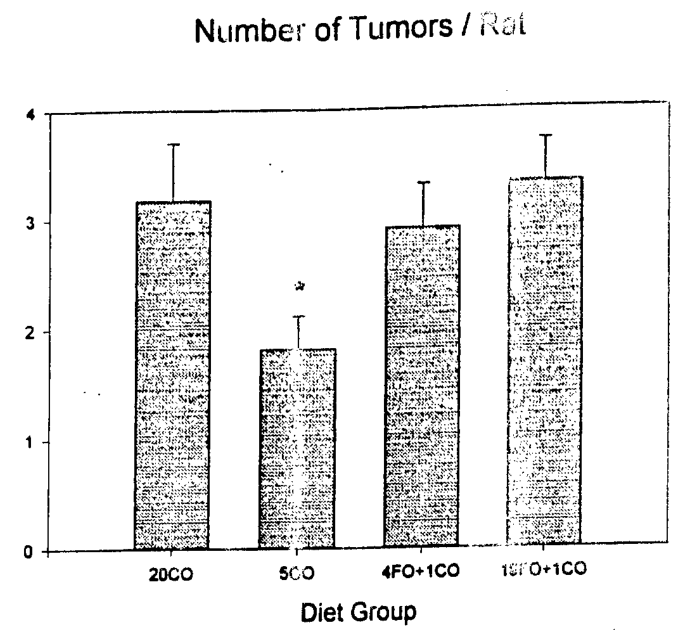


Figure 6. This figure shows the tumor mass (Mean ±S.E.) of each diet group at autopsy.



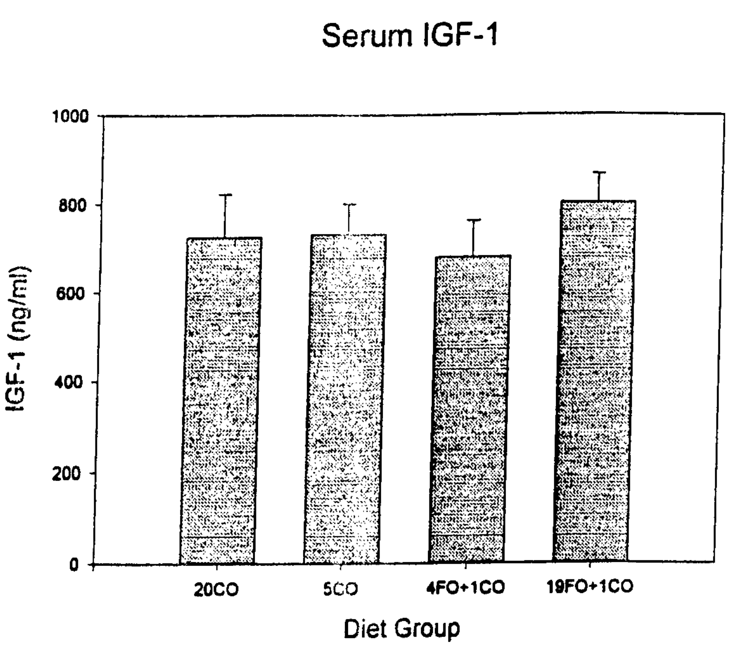
* = $p < 0.05$ vs 20CO

Figure 7. This figure shows the number of tumors (Mean ±S.E.) for each diet group at autopsy.



* = $p < 0.05$ vs 20CO

Figure 8. This figure show the serum IGF-1 value (Mean ±S.E.) for each diet group at autopsy.



Our results demonstrated that the different diets did induce distinctive changes in the tumor fatty acid profiles of each treatment group. The omega-3/omega-6 PUFA ratios of the tumor microsomes of the FO treated were increased relative to the CO treated groups. The data from the R3230AC transplant experiment did show a tendency for the 19%FO+1%CO group to have a reduced tumor burden, but the rapidity of the transplant growth and the small size of the groups prevented these differences from acquiring statistical significance. In the NMU induced tumor study, the low fat groups had a reduced rate of tumor development relative to the 20% CO group, but the tumor characteristics of the 19% FO + 1% CO group did not differ from those of 20% CO group as much as had been previously observed in our laboratory. The reasons for this difference from previous experience were unclear.

In 1996 we undertook another experiment using the NMU induced tumor model. In this study we sought to further evaluate the effects of different blends of omega-3/omega-6 PUFA on tumor development. In particular, we compared the tumorigenic effect of the blend of 15% FO + 5% CO to that of 20% CO and 19% FO + 1% CO. Additionally, because the 1995 experimental results had been less pronounced than those of previous studies using menhaden oil obtained from other sources (e.g. Zapata Haynie Corp., Reedville, VA.), we also examined whether the antioxidants added to menhaden oil had any significant independent effect on tumorigenesis. Tumor parameters from rats fed diets using supplemental antioxidants (a+) were compared to those not supplemented with additional antioxidants (a-). The results of these studies can been seen in the following Figures 9-13. In these figures, all data are presented as the mean ± standard error (SEM). Those differences that are statistically significant ($p<0.05$) from the 20% CO (a-) group are identified with an asterisk (*), while those differences that are statistically significant ($p<0.01$) are marked (**).

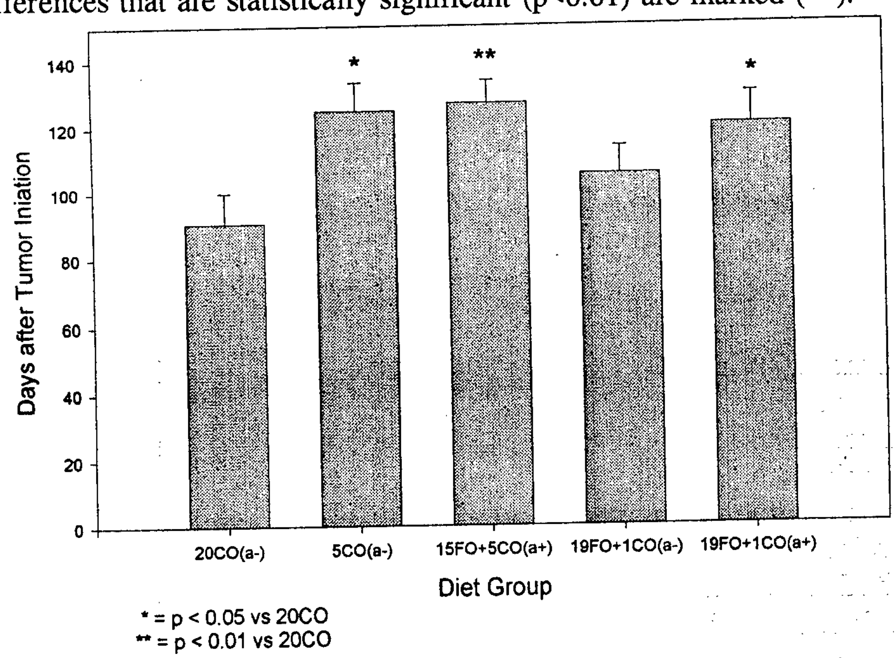


Figure 9. This figure shows the time from NMU administration to first tumor appearance in each diet group (Mean ±S.E.).

Figure 10. This figure shows the time from NMU administration to time of sacrifice for each diet group (Mean ± S.E.).

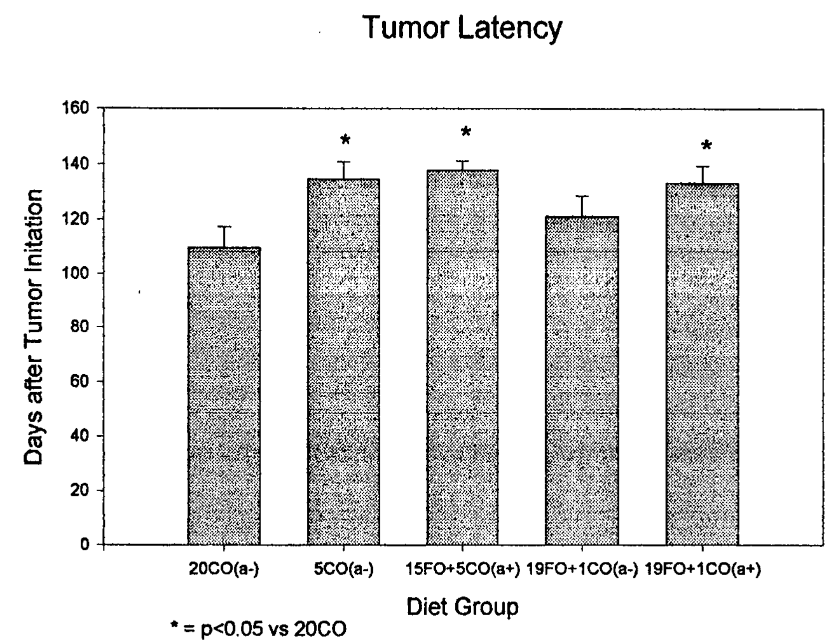


Figure 11. This figure shows the tumor mass (Mean ±S.E.) of each diet group at autopsy.

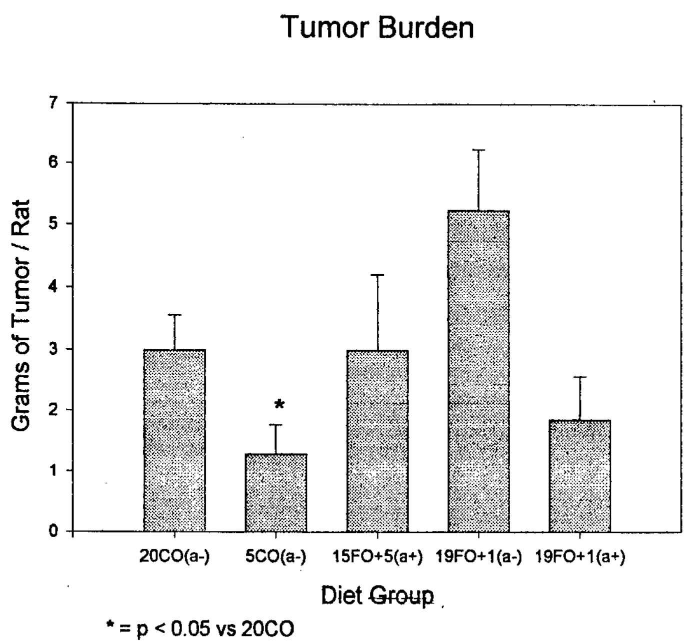
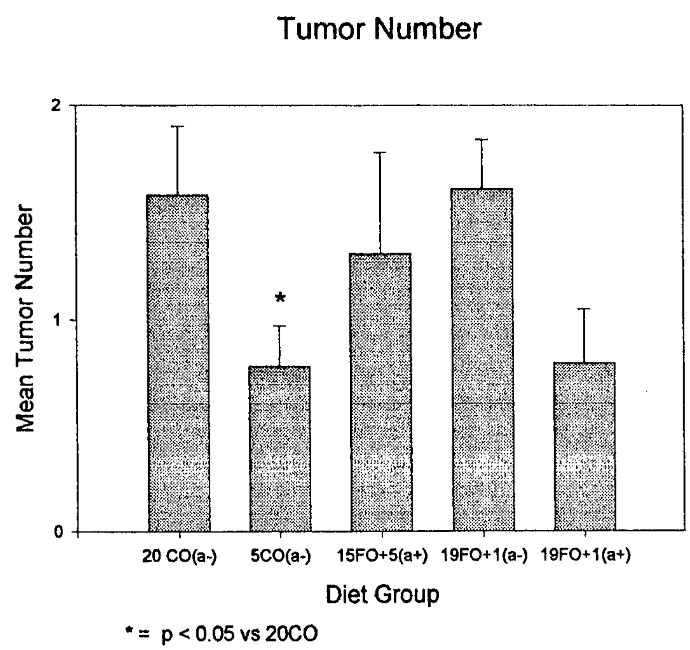


Figure 12. This figure shows the number of tumors (Mean ±S.E.) for each diet group at autopsy.



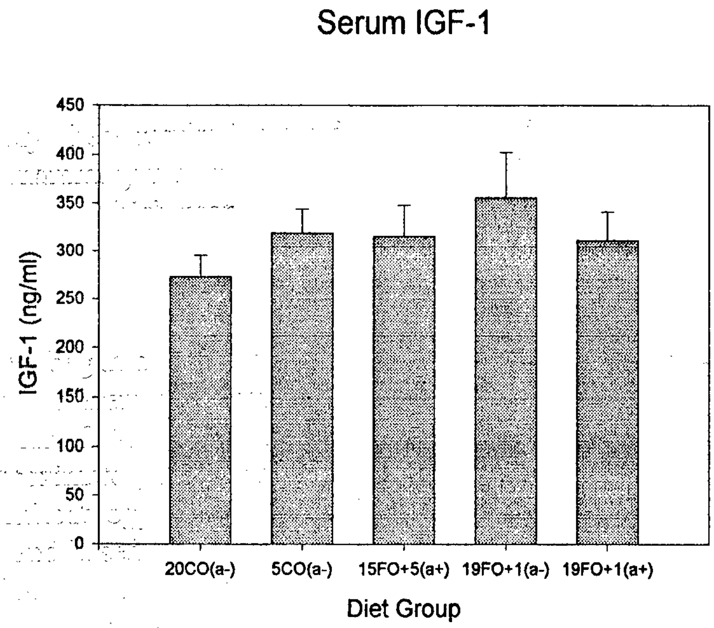


Figure 13. This figure show the serum IGF-1 value (Mean ±S.E.) for each diet group at autopsy.

Collectively, these results indicated that the low fat and the high omega-3 PUFA diets did not enhance mammary tumor development as effectively as high omega-6 PUFA diets. The blended diet with a high proportion of omega-3 PUFA also appeared effective in prolonging tumor latency. There was no significant correlation between the serum IGF-1 levels of the rats at their time of death and either the diet composition or tumor development.

In 1997 we began our molecular biology studies on the RNA derived from the tumors obtained from the different diet treatment groups. Specifically, we compared the content of messenger RNA for IGF-1, IGF-2, the IGF-1 receptor, and the IGF-2 receptor in mammary tumors from approximately forty individual animals. The RNA was extracted from each tumor using a methodology involving TRI Reagent (Molecular Research Center, Inc, Cincinnati, OH) and based on the original methodology of Chomczynski and Sacchi (1). The concentration of RNA was then determined by spectrophotometry, and individual samples stored at -20 deg. C until used in the RNase protection assay. Appropriate probes for the desired messages were obtained from the following investigators: rat IGF-1(Derek LeRoith); rat IGF-2 (Matt Reichler): rat IGF-1 receptor (Derek LeRoith); rat IGF-2 receptor (Derek LeRoith). These probes were then grown in competent cells (E. Coli, DH5a), cloned, and the DNA extracted using the Qiagen Plasmid Purification methodology(Quiagen Inc, Santa Clarita, CA). Labelled RNA transcripts were synthesized "in vitro" using MAXIscript (Ambion, Inc., Austin, TX) methodology. These labeled transcripts were then hybridized with sample RNA, and digested with RNase using the Ribonuclease Protection Assay (Ambion, Inc., Austin, TX) methodology. The protected RNA was run on a polyacrylamide gel and the gel then transferred to filter paper. This was then exposed to X-ray film for radiographic analysis. In some circumstances the film was marked so that appropriated segments of the gel could be subsequently cut out and counted in a scintillation counter.

When we compared our results for these four messenger RNAs, we were unable to find any significant differences amongst the tumors from the different diet groups. It was observed, however, that the IGF-2 receptor message was consistently present in the greatest quantity in these tumors; and although not statistically significant, the data indicated that there was a tendency for the IGF-2 receptor message to be greatest in the tumors from the 20% CO group.

CONCLUSIONS:

We believe the data demonstrate that our dietary interventions did induce correspondent changes in the fatty acid profiles in the tumors from the different treatment groups and associated tumor development. The R3230AC transplant data did show a tendency for the 19%FO+1%CO group to have a reduced tumor burden, but the rapidity of the transplant growth and the small size of the groups did not allow us to achieve statistical significance. The explanation for the relatively limited differences in tumorigenesis among the diet interventions in the 1995 studies with the NMU treated animals is difficult to interpret; but the studies from 1996 appeared to reaffirm our previous conclusion (2) that neither low fat and high omega-3 PUFA diets enhance mammary tumor development as effectively as high omega-6 PUFA diets. The data also lead us to conclude that blended diets with a high proportion of omega-3 PUFA can also prolong tumor latency.

We have concluded from our data that the dietary fat alterations studied were not associated with any significant change in serum levels of IGF. This conclusion appeared consistent with our older observations (3) that there was no difference in "in vitro" synthesis of growth hormone by pituitaries from rats on different quantitative omega-6 PUFA diets despite significant diet induced differences in mammary tumor development. This would also suggest that the liver's ability to synthesize IGF-1 in response to growth hormone is not modified by alterations in dietary fat. There was no attempt was made in these studies to measure the effects of our diets on the serum levels of IGF binding proteins or the circulating levels of IGF-2.

Our molecular biological studies have led us to conclude that we cannot provide any definitive evidence to support the hypothesis that the differences in tumor development associated with variations in dietary fat are due to alterations in the nucleotide messages for the IGFs and their respective receptors in the tumor cells and stroma. There did, however appear to be some possibility the IGF-2 receptor message was slightly enhanced in the tumors of the 20% CO group. The negative nature of our conclusion, continues to leave open the possibility that our studies were limited by our sample size or techniques, but it may also indicate that other possible mechanisms such as post transnational modifications, messenger RNA turnover, or expression of IGF binding proteins could be alternative points of dietary regulation. At this point, however, we would have to conclude that established effects of dietary lipids on eicosanoid metabolism would seem to continue to represent the most important mechanism by which omega-3 and omega-6 dietary lipids influence mammary tumor development.

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